Aspartyl protease inhibitor as a nematode allergen

TECHNICAL FIELD

The present invention relates to novel allergens. The present invention is broadly directed to novel nematode allergens (polypeptides) and associated amino acid and nucleotide sequences including functional fragments and variants thereof. The invention broadly concerns allergens obtained from, nematodes of the superfamily *Trichostrongyloidea*. In particular, the present invention concerns the use of the above molecules to identify animals that are naturally resistant to parasitic nematodes to assist with selective breeding of animals that are innately more disposed to develop immune resistance to, in particular but by no means exclusively, nematode infections in sheep and cattle.

BACKGROUND TO THE INVENTION

The molecules of the present invention are most preferably, but not necessarily exclusively, derived from *Ostertagia circumcincta* (*Teladorsagia circumcincta*) and *Trichostrongylus colubriformis* and have application in reducing the impact of these nematodes or similar nematodes on animals as discussed herein. For ease of reference only the background to the present invention will now be described in relation to *Trichostrongylus colubriformis* infection. However, it will be appreciated by those skilled in the art that such description is also of general application to most nematodes of the superfamily *Trichostrongyloidea*.

Trichostrongylus colubriformis is a nematode parasite which infects the small intestine of sheep and is of significant economic importance in New Zealand and many other countries worldwide. These parasites have a simple life cycle consisting of free-living stages on pasture (egg to infective larvae, L3), and after ingestion, develop through L4-L5-adult in the host gastrointestinal tract. They do not have a tissue migratory phase. *T. colubriformis* live in mucus covered tunnels eroded on the surface of duodenal and intestinal villi.

Presently the only effective means of controlling *T. colubriformis* and other nematodes that infect sheep is through the use of anthelmintics.

Sheep can develop natural immunity to *T. colubriformis* following repeated natural infections. Development of immunity to nematode establishment requires at least seven weeks of continuous infection (Dobson et al 1990). Thus, in the past sheep were effectively immunised by two or three periods of truncated infections, each followed by a treatment with an anthelmintic to remove infection 10-14 days later.

However, a major drawback with conventional anthelmintics is that nematode resistance to a broad spectrum of anthelmintics is now becoming increasingly more widespread and is therefore of serious concern. (Waller, 1997; Sangster *et al*, 1999; Van Wyk *et al*, 1999).

Various studies have suggested that immunity to *T. colubriformis* involve the development of Th2-type immune responses involving hypersensitivity type events. These responses include increased mast cell, globule leucocytes and eosinophil numbers in the intestinal mucosa. Elevated serum IgE levels have also been associated with immunity to nematode infections (Shaw et al., 1998a & b). One possible role of parasite-specific IgE in resistance to nematode infection is its involvement in immediate hypersensitivity reactions, where cross-linking of specific IgE on mucosal mast cells by antigen promotes their degranulation, which in sheep results in the formation of globule leukocytes. The resultant release of inflammatory mediators is considered to be a major mechanism involved in the rejection of parasites in immune sheep. The release of inflammatory mediators may cause a deterioration of the intestinal microenvironment and consequent expulsion of nematode parasites. The inflammatory response may also allow the leakage of immunoglobulins into the intestinal lumen and which may also assist in the expulsion of nematodes from the gastrointestinal tract.

Antigens that elicit hypersensitivity reactions by binding to IgE (i.e. allergens) are therefore useful in diagnostic assays to identify immune animals with a Th2-type hypersensitivity immune response. Thus, such antigens are useful in selective breeding programs for producing animals that are resistant to nematodes.

A major novel allergen of *Trichostrongylus colubriformis*, Tco-Aspin with a molecular weight of 31 kDa, pI 5.1 on 2-D electrophoresis gels has been identified by the inventors and its nucleotide and polypeptide sequence determined. It shares a degree of homology with a

number of proteins identified as nematode aspartyl protease inhibitors. From *Onchocerca volvulus* an aspartyl protease inhibitor-like protein has been cloned by a number of investigators. Ov33-3 (Lucius, et al., 1988) an immunodominant antigen recognised by 96% of onchocerciasis patients; Oc3.6 (Chandrashekar, et al., 1991); OvD5B (Celine Nkenfou, Thesis, "Molecular Cloning of Genes Coding Antigens Specific For Onchocerca volvulus: Evaluation of Expressed Proteins For Use In The Diagnosis Of Onchocerciasis" University of Cameroon (1993). Orthologs have been found in *Brugia malayi* (Bm33) (Dissanayake 1993), *Acanthocheilonema viteae* (Av33) (Willenbucher, et al., 1993), *Dirofilaria immitis* (DiT33) (Maja, et al., 1994, Frank et al., 1998), *Parelaphostrongylus tenuis* (Ptpi) (Duffy et al., 2002) and *Ostertagia ostertagi* (Claerebout et al.,2002) .

Some of these have been used successfully to diagnose important filarial infections of humans including *O. volvulus* (Chandrashekar, et al., 1991 & 1996) and *B. malayi* (Chandrashekar, et al., 1994; Dissanayake , et al., 1993).

However, at present there remains a need to identify allergens for nematode infections in animals, particularly sheep and cattle, and which can be used to diagnose those animals which are naturally inclined to develop immune resistance to nematode infection. Thus, the existence of such allergens may be useful in selective breeding programs for animals innately more disposed to develop immune resistance.

It is an object of the present invention to address the foregoing problems or at least to provide the public with a useful choice.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this

reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

SUMMARY OF INVENTION

The present invention is broadly directed to novel nematode allergens (polypeptides) and associated amino acid and nucleotide sequences including functional fragments and variants thereof to identify animals that are capable of becoming naturally resistant to nematodes. In particular, the present invention concerns the use of these molecules to, assist in selective breeding of animals capable of becoming naturally resistant to nematode infections. More, particularly, the invention is directed towards the use of nucleic acid molecules and polypeptides, which are the same or functional variants of, aspartyl protease inhibitors: (Tco-Aspin), of *Trichostrongylus colubriformis*; and/or (Oc-Aspin), of *Ostertagia circumcincta;* and/or (Hc-Aspin) *Haemonchus contortus* in identifying animals that are innately more disposed to develop immune resistance to nematodes.

The inventors have identified a number of IgE binding antigens (allergens) of *T. colubriformis, O.circumcinta* and *H.contortus,* using western blotting techniques with purified IgE from various immune animals. Using 2-D electrophoresis, the target allergens have been isolated and identified using mass spectrometry. Recombinant allergen proteins were prepared and tested for their ability to bind IgE from the serum of animals with a history of nematode infection.

DISCLOSURE OF INVENTION

According to a first aspect of the present invention there is provided an isolated polypeptide comprising:

a) an amino acid sequence as set forth in any one of SEQ ID NOs. 1, 3, 5 or 7; or

b) a functional fragment or variant of the polypeptides in a) above, wherein the fragment or variant provokes a humoral and/or cellular immunological response in an animal with similar characteristics to that produced by a polypeptide of as outlined in a) above.

According to a second aspect of the present invention there is provided an isolated polypeptide substantially as described above wherein the functional fragment or variant incorporates a B cell or T cell epitope of the polypeptide.

In general the polypeptide may be derived from a nematode parasite of the superfamily Trichostrongyloidea. However, it should also be appreciated that the polypeptide may also be artificially synthesized for example recombinantly synthesized

In particular, the polypeptide and/or nucleic acid molecule may be selected from a nematode parasite from the genera consisting of *Trichostrongylus, Cooperia, Dictyocaulus, Haemonchus, Ostertagia* or *Teladorsagia*.

Preferably, the nematode parasite maybe of the genera *Trichostrongylus* or *Ostertagia* preferably selected from the species consisting of either *T. colubriformis* or *O. circumcincta (Teladorsagia circumcincta)*.

In a third aspect of the present invention there is provided an isolated nucleic acid molecule wherein the molecule:

a) comprises a nucleotide sequence as set forth in any one of SEQ ID NOs. 2, 4, 6 or 8;

b) is a functional fragment or variant of the molecule(s) in a); or

c) is able to hybridise under stringent conditions to the molecule(s) in a) or b); or

d) is a complement of the molecule(s) defined in a), b) or c); or

e) is an anti-sense sequence corresponding to any of the sequences in a) – d).

In a fourth aspect of the present invention there is provided an isolated nucleic acid molecule encoding a polypeptide substantially as described above.

In a fifth aspect of the present invention there is provided a vector or construct comprising the nucleic acid molecule of the present invention.

In a sixth aspect of the present invention there is provided a host cell which has been transformed with a vector or construct of the present invention.

In an seventh aspect of the present invention there is provided an isolated ligand which binds to a polypeptide of the present invention.

In a eighth aspect of the present invention there is provided a probe capable of hybridizing under stringent conditions to a nucleic acid molecule of the present invention.

In a ninth aspect of the present invention there is provided a probe for a polypeptide substantially as described above.

In a tenth aspect of the present invention there is provided a probe for a ligand substantially as described above when said ligand is bound to a polypeptide of the present invention.

In an eleventh aspect of the present invention there is provided a method for determining the immune status of an animal to a nematode infection characterized by steps of:

a) obtaining a blood or serum sample from the animal;

b) preparing an IgE enriched or IgG depleted preparation of the sample in a);

c) contacting the sample at a) with a polypeptide comprising the amino acid sequence of any one of SEQ ID NOs. 1, 3, 5 or 7 or a functional fragment or variant thereof;

d) contacting the preparation from c) with a probe for the immuno-complex formed by IgE and the polypeptide;

e) detecting the probe to identify the immune status of the animal by the presence or absence of the probe.

In a twelfth aspect of the present invention there is provided a method for determining the immune status of an animal to a nematode infection characterized by steps of:

a) obtaining a blood or serum sample from the animal

b) preparing an IgE enriched or IgG depleted preparation of the sample in a);

c) exposing the preparation from b) with a polypeptide comprising the amino acid sequence of any one of SEQ ID NOs. 1, 3, 5 or 7 or a functional fragment or variant thereof ;

d) washing the preparation from c) to remove any unbound IgE (i.e. IgE that is not bound to the polypeptide);

e) detection of immuno-complex formed by the polypeptide and IgE at step c) with monoclonal antibodies to IgE.

f) detection of IgE with appropriately labeled anti-antibodies.

Preferably, at step b) the blood or serum is IgE enriched or IgG depleted using ammonium sulphate precipitation or affinity purification of sheep IgE. However, this should not be seen as limiting.

Preferably, the exposure step c) may be performed by coating the polypeptide of SEQ ID NO. 1 onto microtiter plates. However, other suitable methods for exposing are of course envisaged.

In preferred embodiments, the monoclonal antibodies of step d) may be mouse monoclonal antibodies. However, antibodies sourced from other animals may also be used without departing from the scope of the present invention.

Preferably, the sample may be exposed to the polypeptide via an enzyme-linked immunoassay (ELISA) or other suitable type of assay.

According to a thirteenth aspect there is provided a method of determining the immune status of an animal comprising the steps of:

a) exposing a portion of the animal's skin to a polypeptide of the present invention;

b) determining the immune status by the presence or absence of an immune or allergic reaction.

According to a fourteenth aspect there is provided a method for selectively breeding animals resistant to nematode infection characterized by steps of:

a) determining the immune status of male and female animals;

b) selecting males and females disposed to develop immune resistance to nematodes;

c) using selected animals to breed progeny resistant to said infection.

In general, the animal may be selected from sheep, goats, cattle and equines. However, this list should not be seen as limiting as the animal may be any mammal which is prone to infection with nematode parasites.

Preferably the animal may be selected from either cattle or sheep. Most preferably the animal may be a sheep.

In a fifteenth aspect of the present invention there is provided an isolated polypeptide substantially as described above wherein the polypeptide is a functional fragment or variant of SEQ ID NO. 5 having at least 90% homology to SEQ ID NO. 5.

In a sixteenth aspect of the present invention there is provided an isolated nucleic acid molecule substantially as described above wherein the molecule is a functional fragment or variant of SEQ ID NO. 6 having at least 94% homology to SEQ ID NO. 6.

In a seventeenth aspect of the present invention there is provided an isolated polypeptide substantially as described above wherein the polypeptide is a functional fragment or variant of SEQ ID NO. 1 having at least substantially 75% homology to SEQ ID NO. 1.

In an eighteenth aspect of the present invention there is provided an isolated nucleic acid molecule substantially as described above wherein the molecule is a functional fragment or variant of SEQ ID NO. 2 having at least substantially 70% homology to SEQ ID NO. 2.

In a nineteenth aspect of the present invention there is provided an isolated polypeptide substantially as described above wherein the polypeptide is a functional fragment or variant of SEQ ID NO. 3 having at least 80% homology to SEQ ID NO. 3.

In a twentieth aspect of the present invention there is provided an isolated nucleic acid molecule substantially as described above wherein the molecule is a functional fragment or variant of SEQ ID NO. 4 having at least substantially 70% homology to SEQ ID NO. 4.

In a twenty-first aspect of the present invention there is provided an isolated polypeptide substantially as described above wherein the polypeptide is a functional fragment or variant of SEQ ID NO. 7 having at least 80% homology to SEQ ID NO. 7.

In a twenty-second aspect of the present invention there is provided an isolated nucleic acid molecule substantially as described above wherein the molecule is a functional fragment or variant of SEQ ID NO. 8 having at least 75% homology to SEQ ID NO. 8.

It is to be clearly understood that the invention also encompasses peptide analogues, which include but are not limited to the following:

1. Compounds in which one or more amino acids is replaced by its corresponding D-amino acid. The skilled person will be aware that retro-inverso amino acid sequences can be synthesised by standard methods; see for example Chorev and Goodman, 1993;

2. Peptidomimetic compounds, in which the peptide bond is replaced by a structure more resistant to metabolic degradation. See for example Olson *et al*, 1993; and

3. Compounds in which individual amino acids are replaced by analogous structures for example, gem-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge.

The use of such alternative structures can provide significantly longer half-life in the body, since they are more resistant to breakdown under physiological conditions.

Methods for combinatorial synthesis of peptide analogues and for screening of peptides and peptide analogues are well known in the art (see for example Gallop et al, 1994; Hogan, 1997).

For the purposes of this specification, the term "peptide and peptide analogue" includes compounds made up of units which have an amino and carboxy terminus separated in a 1,2, 1,3, 1,4 or larger substitution pattern. This includes the 20 naturally-occurring or "common" α-amino acids, in either the L or D configuration, the biosynthetically-available or "uncommon" amino acids not usually found in proteins, such as 4-hydroxyproline, 5-hydroxylysine, citrulline and ornithine; synthetically-derived α-amino acids, such as α-methylalanine, norleucine, norvaline, Cα- and *N*-alkylated amino acids, homocysteine, and homoserine; and many others as known in the art.

It also includes compounds that have an amine and carboxyl functional group separated in a 1,3 or larger substitution pattern, such as β-alanine, γ-amino butyric acid, Freidinger lactam (Freidinger *et al*, 1982), the bicyclic dipeptide (BTD) (Freidinger *et al*, 1982; Nagai and Sato, 1985), amino-methyl benzoic acid (Smythe and von Itzstein, 1994), and others well known in the art. Statine-like isosteres, hydroxyethylene isosteres, reduced amide bond isosteres, thioamide isosteres, urea isosteres, carbamate isosteres, thioether isosteres, vinyl isosteres and other amide bond isosteres known to the art are also useful for the purposes of the invention.

A "common" amino acid is a L-amino acid selected from the group consisting of glycine, leucine, isoleucine, valine, alanine, phenylalanine, tyrosine, tryptophan, aspartate, asparagine, glutamate, glutamine, cysteine, methionine, arginine, lysine, proline, serine, threonine and histidine. These are referred to herein by their conventional three-letter or one-letter abbreviations.

An "uncommon" amino acid includes, but is not restricted to, one selected from the group consisting of D-amino acids, homo-amino acids, N-alkyl amino acids, dehydroamino acids, aromatic amino acids (other than phenylalanine, tyrosine and tryptophan), ortho-, meta- or para-aminobenzoic acid, ornithine, citrulline, norleucine, α-glutamic acid, aminobutyric acid (Abu), and α-α disubstituted amino acids.

The term "polypeptide", "peptide" and "protein" are all used interchangeably herein to refer to a molecule comprising a chain of one or more amino acids.

The term "variant" as used herein refers to nucleotide and polypeptide sequences wherein the nucleotide or amino acid sequence exhibit:

- at least substantially 70%, or at least substantially 75% homology with the nucleotide or amino acid sequences contained in the sequence listing;
- preferably exhibits at least substantially 80% or 85% homology or greater with said sequences; and
- most preferably exhibits a homology selected from substantially 90-99% homology to the sequences contained in the sequence listing and which may include at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% homology to said;

as assessed by GAP or BESTFIT (nucleotides and peptides), or BLASTP (peptides) or BLASTN (nucleotides). The variant may result from modification of the native nucleotide, or amino acid sequence, by such modifications as; insertion, substitution or deletion of one or more nucleotides or amino acids, or it may be a naturally-occurring variant.

Thus, the term variant should be taken to include changes (i.e. conservative substitution) to the nucleotide sequences set forth herein which do not alter the amino acid being coded for, due to the degenerate nature of the genetic code. The term "variant" also includes homologous sequences which hybridise to the sequences of the invention under standard, but most preferably under stringent conditions.

In general "stringent conditions" for determining the degree of homology may refer to:

a) low salt concentrations (i.e. less than 1M, preferably less than 500mM and most preferably less that 200mM); and

b) high hybridization temperatures (i.e. at least 30°C, preferably greater than 37°C and most preferably greater than 50°C).

However, as the stringency of hybridization can be affected by other factors including probe composition and the presence of organism solvents, it is the combination of parameters above that, which is important in determining stringency.

For example, stringent hybridization conditions can be defined as 2 x SSC at 65°C, or and for example standard hybridization conditions can be defined as 6 x SCC at 55°C, provided always that the variant is capable of diagnosing nematode infection. Where such a variant is desired, the nucleotide sequence of the native DNA is altered appropriately. This alteration can be effected by synthesis of the DNA or by modification of the native DNA, for example, by site-specific or cassette mutagenesis. Preferably, where portions of cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed, using techniques standard in the art.

The term "isolated" means substantially separated or purified away from contaminating sequences in the cell or organism in which the nucleic acid naturally occurs and includes nucleic acids purified by standard purification techniques as well as nucleic acids prepared by recombinant technology, including PCR technology, and those chemically synthesised. Preferably, the nucleic acid molecule is derived from genomic DNA or the mRNA of the *Trichostrongylus colubriformis*.

The nucleic acid molecule may be an RNA, cRNA, genomic DNA or cDNA molecule, and may be single- or double-stranded. The nucleic acid molecule may also optionally comprise one or more synthetic, non-natural or altered nucleotide bases, or combinations thereof.

A fragment of a nucleic acid is a portion of the nucleic acid that is less than full length and comprises at least a minimum sequence capable of hybridising specifically with a nucleic acid molecule according to the present invention (or a sequence complementary thereto) under stringent conditions as defined below. A fragment of a polypeptide is a portion of the polypeptide that is less than full length but which still retains a biological function of being

capable of diagnosing nematode infection. A fragment according to the invention has at least one of the biological activities of the nucleic acid or polypeptide of the invention.

The polypeptides of the invention can be prepared in a variety of ways. For example, they can be produced by isolation from a natural source, by synthesis using any suitable known techniques (such as by stepwise, solid phase, synthesis described by Merryfield (1963), *J.Amer.Chem.Soc.* Vol 85:2149-2156) or as preferred, through employing DNA techniques.

The cloning vector may be selected according to the host or host cell to be used. Useful vectors will generally have the following characteristics:

(a) the ability to self-replicate;

(b) the possession of an appropriately positioned single target for any particular restriction endonuclease; and

(c) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

Two major types of vector possessing these characteristics are plasmids and bacterial viruses (bacteriophages or phages). Presently preferred vectors include the bacteriophage lambda Uni-ZAP™ XR and the modified plasmid pBAD18 vector, AY2-4. Tight regulation, modulation, and high-level expression are characteristics of vectors containing the arabinose PBAD promoter. *J. Bacteriol.* 177:4121-4130).

The DNA molecules of the invention may be expressed by placing them in operable linkage with suitable control sequences in a replicable expression vector. Control sequences may include origins of replication, a promoter, enhancer and transcriptional terminator sequences amongst others. The selection of the control sequence to be included in the expression vector is dependent on the type of host or host cell intended to be used for expressing the DNA.

Generally, eucaryotic, yeast, insect or mammalian cells are useful hosts. Also included within the term hosts are plasmid vectors. Suitable procaryotic hosts include *E. coli*, *Bacillus* species and various species of *Pseudomonas*. Commonly used promoters such as β-lactamase (penicillinase) and lactose (lac) promoter systems are all well known in the art. Any available

promoter system compatible with the host of choice can be used. Vectors used in yeast are also available and well known. A suitable example is the 2 micron origin of replication plasmid.

Similarly, vectors for use in mammalian cells are also well known. Such vectors include well known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences, Herpes simplex viruses, and vectors derived from a combination of plasmid and phage DNA.

Further eucaryotic expression vectors are known in the art (e.g. P.J. Southern and P.Berg, *J. Mol. Appl. Genet.* 1 327-341 (1982); S. Subramani et al., *Mol.Cell.Biol.* 1, 854-864 (1981); R J. Kaufmann and P.A. Sharp, "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reducase Complementary DNA Gene, *J. Mol. Biol.* 159, 601-621 (1982); R J. Kaufmann and P.A. Sharp, *Mol.Cell.Biol.* 159, 601-664(1982); S.I. Scahill et al., "Expressions And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc. Natl. Acad. Sci. USA.* 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci. USA.* 77, 4216-4220, (1980).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the glycolytic promoters of yeast acid phosphatase, e.g. Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g. the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic and eucaryotic cells and their viruses or combinations thereof.

A preferred promoter for use herein is the arabinose promoter (Guzman, L., Belin, D., Carson, M. J. and Beckwith, J.,1995. Ref), however, any suitable promoter is included within the scope of the present invention as would be appreciated by a skilled worker.

In the construction of a vector it is also an advantage to be able to distinguish the vector incorporating the foreign DNA from unmodified vectors by a convenient and rapid assay. Reporter systems useful in such assays include reporter genes, and other detectable labels which produce measurable colour changes, antibiotic resistance and the like. In one preferred vector, the β-galactosidase reporter gene is used, which gene is detectable by clones exhibiting a blue phenotype on X-gal plates. This facilitates selection. In one embodiment, the β-galactosidase gene may be replaced by a polyhedrin-encoding gene; which gene is detectable by clones exhibiting a white phenotype when stained with X-gal. This blue-white color selection can serve as a useful marker for detecting recombinant vectors.

The term "ligand" refers to any molecule which can bind to another molecule such as a polypeptides or peptide, and should be taken to include, but not be limited to, antibodies, cell surface receptors or phage display molecules.

The term "phage display molecule" refers to a molecule, such as a peptide, protein or antibody; expressed on the cell surface of a bacteriophage or like microorganism, by techniques well known in the art.

It should be appreciated that the term "antibody" encompasses fragments or analogues of antibodies which retain the ability to bind to a polypeptide of the invention, including but not limited to Fr, $F(ab)_2$ fragments, scFv molecules and the like. The antibody may be polyclonal but is preferably monoclonal. In some embodiments the ligand may be a phage display molecule.

"Primers" are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length, which are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, preferably a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g. by the polymerase chain reaction (PCR) or other nucleic acid amplification methods well known in the art. PCR-primer pairs can be derived from the sequence of a nucleic acid according to the present invention, for example, by using

computer programs intended for that purpose such as Primer (Version 0.5© 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

"Probes" as used herein refers to a ligand which is labelled in some way (for example, radioactively, fluorescently or immunologically), and which are used to find and mark a target molecule by binding to the target molecule or a molecule associated therewith. In general, the target molecule may be a nucleic acid or polypeptide of the present invention, it may also include a polypeptide of the present invention when complexed (e.g. bound) to another ligand (such as for example an antibody), or the ligand when bound (i.e. associated with) the polypeptide.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

The present invention will now be described in detail, by way of reference only, to the following non-limiting examples.

BRIEF DESCRIPTION OF SEQUENCE LISITING

Further aspects of the invention will be apparent from the attached sequence listing, in which:

SEQ ID NO. 1 shows the amino acid sequence of *T.colubriformis* Aspin (Tco-Aspin)

SEQ ID NO. 2 shows the nucleotide sequence of *T.colubriformis* Aspin (Tco-Aspin)

SEQ ID NO. 3 shows the amino acid sequence of a truncated *T.colubriformis* Aspin (Tco-Aspin*)

SEQ ID NO. 4 shows the nucleotide sequence of a truncated *T.colubriformis* Aspin (Tco-Aspin*)

SEQ ID NO. 5 shows the amino acid sequence of *O.circumcincta* () Aspin (Oc-Aspin)

SEQ ID NO. 6 shows the nucleotide sequence of *O.circumcincta* Aspin (Oc-Aspin)

SEQ ID NO. 7 shows the amino acid sequence of *H.contortus* Aspin (Hc-Aspin)

SEQ ID NO. 8 shows the nucleotide sequence of *H.contortus* Aspin (Hc-Aspin)

BRIEF DESCRIPTION OF DRAWINGS

Further aspects of the present invention will become apparent from the following description which is given by way of example only and with reference to the accompanying drawings in which:

Figure 1 shows one-dimensional immunoblot analysis of *T. colubriformis* L3 homogenate proteins probed with time-course IgE samples from sheep during the development of immunity to nematode infection. IgE was purified from serum samples taken at various time-points following weaning of lambs in the field out to 18 months of age. Arrows indicate major antigen bands detected on immunoblots (I- 42 kDa, II- 31-32 kDa, III- 27-31 kDa, IV- 20-21 kDa, V- 2-14 kDa).

Figure 2 shows two-dimensional immunoblot analysis of *T. colubriformis* L3 homogenate proteins. Proteins were subjected to isoelectric focusing (left to right) followed by SDS-PAGE (top to bottom). Immunoblot was probed with affinity purified total IgE antibodies from pooled sera from immune sheep. Spot corresponding to Tco-Aspin is indicated by a circle and Tco-Aspin breakdown product by a square.

Figure 3 shows two-dimensional gel electrophoresis separation of *T. colubriformis* L3 homogenate total proteins Coomassie blue stained. Spot corresponding to Tco-Aspin is indicated by a circle and Tco-Aspin breakdown product by a square.

Figure 4 shows the alignment of Tco-Aspin with other members of the putative aspartyl protease inhibitor family. Database accession numbers for the previously sequenced members are: (Oo) *Ostertagia ostertagi* (CAD10783), (Pt) *Parelaphostrongylus tenuis* (AAG50205), (Ce) *Caenorhabditis elegans* (AAC46663), (Av) *Acanthocheilonema viteae* (S23229), (Di) *Dirofilaria immitis*

(AAA70419), (Ov) *Onchocerca* volvulus (AAA29419). Alignment begins at the putative initiating methionines. The conserved residues RDL of putative YVRDLT sequence motif suggested to be critical for inhibitor function are shown by *. Invariant cysteine residues are marked by ^.

Figure 5 shows one dimensional immunoblot of *T. colubriformis* adult homogenate (lane 1) and L3 homogenate (lane 2) immunostained with rabbit anti Tco-Aspin sera.

Figure 6 shows two-dimensional immunoblot analysis of *T. colubriformis* L3 homogenate proteins. Proteins were subjected to isoelectric focusing (left to right) followed by SDS-PAGE (top to bottom). Immunoblot was probed with serum from rabbit immunized with purified recombinant Tco-Aspin.

Figure 7 shows one-dimensional SDS-PAGE gel of affinity purified native Tco-Aspin stained with silver stain and corresponding immunoblot developed with rabbit anti Tco-Aspin sera.

Figure 8 shows one-dimensional SDS-PAGE gel transferred to PVDF of affinity purified native Tco-Aspin stained with Coomassie blue with segments excised for Edman sequencing (A) and corresponding immunoblot developed with rabbit anti Tco-Aspin sera (B)

EXPERIMENTAL

EXAMPLE 1

Nematode larvae and antigen preparation

Infective larvae (3rd stage) of *T. colubriformis* (TcL3) were obtained from cultures of faeces taken from monospecifically infected Romney sheep. Somatic antigen (TcL3-Homog) was prepared by homogenising exsheathed larvae under liquid nitrogen in a mortar and pestle. Soluble protein was extracted in 5 mM Tris buffer pH 7.6 with protease inhibitor (Complete, Boehringer Mannheim) added. After centrifugation at 3000 rpm for 10 min, the protein

concentration of the supernatant was determined by absorbance at 230/260 nm, before being aliquoted and frozen at -70°C.

EXAMPLE 2

Two-dimensional gel electrophoresis

Sample preparation and isoelectric focusing was performed as described in the manufacturer's instruction manual with slight modifications. Briefly, 900 µl of TcL3-Homog (~360-1920 µg) was precipitated in 3600 µl acetone at -20°C for 30-120 min. After centrifuging at 17000g for 30 min at 4°C, acetone was removed and the precipitate dried. The precipitate was dissolved in ~280 µl of rehydration buffer containing 8 M urea, 3 M thiourea, 4% CHAPS, 40 mM dithiothreitol, 0.5% IPG buffer (pH 3-10 or pH 4-7) and a trace of bromophenol blue. The solution was used to rehydrate Immobiline DryStrips (13 or 18 cm, pH 3-10 or pH 4-7) at 30V for 30-60 hr on an IPGphor (Amersham Pharmacia Biotech). Proteins were focused at 20°C according to the following voltage protocol: 120V for 2 hr, 500V for 1hr, 1000V for 1 hr, 1000-8000V gradient for 30 min, 8000V for 6.0-8.5 hr (Total Vhr were 52000 to 80000 Vhr). After a standard equilibration step, proteins were run in the second dimension on 10 to 18% linear SDS-Page gels. Proteins in the resolved gels were either stained with Copper (Bio-Rad) or microwave assisted Coomassie blue R-250 (Wong 2000) or transferred to nitrocellulose according to the manufacturer's directions (Bio-Rad).

EXAMPLE 3

Western blotting

Proteins transferred to nitrocellulose were initially detected with Ponceau S (Harper & Speicher, 1995) and the membrane marked to assist later identification of proteins in gels for further analysis. All incubations were at room temperature and washing of membranes was with PBS + 0.05% Tween 20, 3 x 5 min. Following blocking with Blotto, nitrocellulose was probed with purified sheep IgE (Shaw et al., 1997) at 10-15 µg ml^{-1} overnight. IgE was obtained from sheep infected monospecifically with T *colubriformis* or field grazing sheep and which showed high levels of immunity as determined by faecal egg count and worm counts (data not shown).

Membranes were incubated sequentially with mouse monoclonal anti-ovine IgE antibodies (XB6 & YD3 1/15 dilution of culture supernatant) and horseradish peroxidase-conjugated goat anti-mouse IgG gamma chain specific (l/1000) (Sigma Chemical) before detection with 3-Amino-9-ethylcarbozole. A protein spot corresponding to a strong IgE binding spot on Western blots with molecular weight 33,000 and pI 5.1 (Tco-Aspin) was analysed to determine amino acid sequence information.

EXAMPLE 4

Protein in-gel digestion

Protein spots from Coomassie blue stained gels were identified by comparison with IgE stained companion Western blots. Spots were excised, destained and digested with trypsin (Shevchenko et al., 1996). Briefly, spots were destained in 25 mM NH_4HCO_3 in 50% acetonitrile (ACN). Spots were then dried by centrifugal evaporation and rehydrated in 25mM NH_4HCO_3 containing 12.5 µg ml-l trypsin and incubated at 37°C for 16 hr. Peptides were then extracted sequentially with 25 mM NH_4HCO_3, 50% ACN/0.5% trifluoroacetic acid (TFA)(3 times) and 100% ACN. The combined extracts were dried in a Speedvac, rinsed with milliQ water, then dried again. Extracts were then dissolved in 0.5%TFA. The tryptic digest was cleaned up with ZipTips (Millipore) according to the manufacturer's directions. Peptides were eluted from ZipTips in matrix solution consisting of a saturated solution of α-Cyano-4-hydroxycinnamic acid in 50% ACN/ 0.5% TFA and spotting directly onto Maldi sample plate.

EXAMPLE 5

Protein identification by peptide mass fingerprinting

Molecular masses of tryptic peptides from each protein spot were determined on a MALDI-tof instrument equipped with a nitrogen laser at 337 nm (Perceptive Biosystems, Voyager mass spectrometer). All MALDI spectra were externally calibrated using CALMIX 2. Peptide masses were submitted for protein mass database searching at ProFound (URL: http://www.proteometrics.com/prowl-cgi/ ProFound.exe).

EXAMPLE 6

De novo peptide sequencing by electrospray ionization

Protein spots were submitted to the Australian Proteome Analysis Facility (APAF, Sydney, Australia) for determination of amino acid sequence from selected peptides. Briefly samples undergo a 16-hour tryptic digest at 37°C. The resulting peptides were purified using a ZipTip to concentrate and desalt the sample. The samples were then analysed by ESI-TOF MS/MS using a Micromass Q-TOF MS equipped with a nanospray source, using either flow injection coupled to a Waters CapLC, or manually acquired using borosilicate capillaries for nanospray acquisition. Data was acquired over the m/z range 400-1800Da to select peptides for MS/MS analysis. After peptides were selected, the MS was switched to MS/MS mode and data collected over the m/z range 50-2000Da with variable collision energy settings. Amino acid sequences were then determined from this data.

EXAMPLE 7

N-terminal amino acid sequencing

Crude T. colubriformis L3 protein was separated by 2-D electrophoresis (as in EXAMPLE 2) and electroblotted to PVDF membrane using standard techniques for preparing proteins for sequencing. The PVDF was stained with 0.1% Ponceau S and or 0.025% Coomassie blue R the spots corresponding to allergens on companion Western blots (EXAMPLE 3) were identified and cut out. PVDF spots were then submitted for Edman degradation analysis to establish the amino acid sequence of the amino terminus for as many residues as could be established.

EXAMPLE 8

Molecular Biology

The protein spot when subjected to QTOF mass spectrometry yielded the sequence of two peptides, TC41A and TC41I. Also the mature amino terminus of the protein was determined by protein sequencing. Forward and reverse degenerate oligonucleotides were designed and used as primers in polymerase chain reaction (PCR) under permissive conditions. *T. colubriformis*

total RNA was prepared with Trizol (GibcoBRL) using manufacturers protocols except the initial extraction was performed by grinding larvae in presence of Trizol under liquid Nitrogen. Total RNA was converted to cDNA by standard procedures using SuperscriptII (GibcoBRL) and used as the template in subsequent PCR. The primers used in PCR are listed below.

SL1 GGTTTAATTACCAAGTTTGA

Tco-Aspin TC41Afor GAACAGCAGGAAATCACCAAYTTAYGARAA

Tco-Aspin TC41Arev TTTTCGTAGTTGGTGATTTCYTGYTGYTC

Tco-Aspin TC41Ifor GGAGARGCTGARCARTT

Tco-Aspin TC41Irev AAYTGYTCAGCYTCNCC

TC41.sig.5.nde GATCGGCGCGCCATATGGCACCAAGACAGAACGC

Tco-Aspin TC41.not.3 GATCTGCGGCCGCATAGATCCTCGTGCAGAAGTT

Tco-Aspin.3.2.not GAATTCGCGGCCGCCTTCTTCTCAAACTCCTTCAACTC

Hc-Aspin 5.1 ATGAAGTTGGTCGTRCTSTGCGTTCTGTGTG

HO-Aspin 3.1 ATAGATCCTTGTGCAAAAGTTGGGC

Hc-Aspin 3.RACE GCATGGTTCAGGGCAACAAGGTC

Hc.Aspin.5.Nde GATCGGCGCGCCATATGCTCACTGTGGGCACGATCTCC

Hc.Aspin.3.Not GATCTGCGGCCGCATAGATTCTCGTACAGAAGTT

Oc.Aspin.5.Nde GATCGGCGCGCCATATGCTTACTGTGGGCACAATCGCT

Oc.Aspin.3.Not GATCTGCGGCCGCATAGATCCTTGTGCAAAAGTTG

PolyTA TTTTTTTTTTTTTTTTTTTTA

PolyTC TTTTTTTTTTTTTTTTTTTTC

PolyTG TTTTTTTTTTTTTTTTTTTTG

PCR reactions were performed with *T. colubriformis*, *H. contortus*, and *O. circumcincta* cDNA with various combinations of these primers, in addition to the nematode spliced leader primer (SL1). PCR conditions using degenerate primers was as follows; 95° for 2', followed by 10 cycles of 95° for 30sec, 35° for 45sec, + 1.0° per cycle, 72° for 45sec followed by 30 cycles of 95° for 30sec, 45° for 45sec, + 0.2° per cycle, 72° for 60sec, + 2sec per cycle. Utilizing *T. colubriformis* cDNA a product of 240bp was observed by agarose gel electrophoresis of products following reactions with the primer combination of SL1 and TC41Arev while all other combinations yielded no detectable PCR product. A product of 740bp was observed by agarose gel electrophoresis of products following reactions with the primer combination of SL1 and HO-Aspin 3.1 with the *O. circumcincta* cDNA template. A product of 700bp was observed by agarose gel electrophoresis of products following reactions with the primer combination of Hc-Aspin 5.1 and HO-Aspin 3.1 with the *H. contortus* template. The 3' end of *H. contortus* Aspin was generated by use of the primers Hc-Aspin 3.RACE and PolyTG in a PCR reaction with the *H. contortus* cDNA template. PCR products were cloned into pCR2.1 using TA cloning (Invitrogen) and sequenced using ABI 377 automated DNA sequencer (Waikato DNA sequencing facility). Analysis of the *T. colubriformis* sequence revealed homology to the 5' end of various nematode protease (pepsin) inhibitor mRNAs. It was also noted that a previously identified EST clone (unpublished) had homology to these protease (pepsin) inhibitors at the 3' end. This allowed the synthesis of Tco-Aspin specific 5' and 3' primers, TC41.not3 and TC41.sig.5.nde, each containing restriction endonuclease sites for cloning into the expression vector AY2-4. These primers were used to generate the mature Tco-Aspin coding sequence in a PCR reaction using *T. colubriformis* cDNA as template. The primers TC41.sig.5.nde and Tco-Aspin.3.2.not were used to generate truncated Tco-Aspin (Aspin*) coding sequence in a PCR reaction using *T. colubriformis* cDNA as template. The primers Hc.Aspin.5.Nde and Hc.Aspin.3.Not were used to generate the mature Hc-Aspin coding sequence in a PCR reaction using *H. contortus* cDNA as template. The primers Oc.Aspin.5.Nde and Oc.Aspin.3.Not were used to generate the mature Oc-Aspin coding sequence in a PCR reaction using *O. circumcincta* cDNA as template. After PCR the products were restricted with the appropriate

restriction enzyme and gel purified (Qiagen). The resultant DNA products were digested b appropriate restriction enzymes, cloned into the expression vector AY2-4 and the cloned insert was sequenced.

Bacteria containing either the Tco-Aspin/ AY2-4, Tco-Aspin*/ AY2-4, Hc-Aspin/ AY2-4 or Oc-Aspin/ AY2-4 construct were grown in LB broth at 37°C to an optical density (600nm) of 0.8 at which time protein synthesis was induced by the addition of 0.2% L(+)arabinose (BDH). Induction proceeded for 16 hr at 30 at which time the induced bacteria were pelleted and resuspended in 300mM NaCl/50mM $P0_4$ pH 8.0 buffer containing 1mg/ml lysozyme and incubated on ice for 1 hr. The bacteria were sonicated and the solution clarified by centrifugation to yield a soluble bacterial fraction. Recombinant protein was immobilized to Ni-NTA resin (Qiagen), washed with 300mM NaCl/50mM $P0_4$ pH 8.0 buffer containing 20mM imidazole to remove the contaminating *E. coli* proteins after which the recombinant protein was eluted by the addition of 100mM imidazole. The recombinant protein was subsequently dialyzed versus 300mM NaCl/50mM $P0_4$ pH 8.0 buffer to remove the imidazole and the protein concentration determined. Recombinant Tco-Aspin was also purified from the insoluble, inclusion body fraction. In this case the bacterial pellet following centrifugation of the lysed bacteria was solubilised in 8M urea, 300mM NaCl/50mM $P0_4$ pH 8.0 buffer and applied to the Ni-NTA column equilibrated in the same buffer. After washing with several column volumes, the column was washed in the same buffer without urea prior to elution in imidazole as above.

EXAMPLE 9

Immunisation of Rabbits

New Zealand white rabbits were immunized with purified 6-his tagged recombinant protein. Immunizing doses consisting of 100 µg of Tco-Aspin mixed with Montanide ISA50 at a ratio of 6 parts Montanide: 5 parts aqueous solution were injected intra muscularly and subcutaneously. Four weeks later a second immunizing dose prepared exactly as the first, was administered by the same route. The rabbit was bled by heart puncture under anesthetic 10 days after the second immunization. After clotting for 1 hour at room temperature the blood was centrifuged

at 1300g for 15 minutes. The serum was collected and stored at –20°C until needed. This serum is referred to as anti Tco-Aspin.

EXAMPLE 10

Affinity purification of native Tco-Aspin

IgG was purified from the sera of a rabbit immunised for Tco-Aspin by Protein G sepharose affinity using standard techniques. Purified antibody was bound to NHS-activated sepharose (Amersham Pharmacia) as per the manufacturer's protocols. TcL3-homog was passed through the immobilised rabbit antibody column at 0.5-2.0 ml/min. The column was washed with buffer (20 mM phosphate buffer, 500 mM NaCl, pH 7.0) until a baseline was reached at absorbance of 280 nm. The bound native Tco-Aspin was eluted with either 0.1M glycine or formic acid pH 3.0. The elutant was neutralised with 1M Tris or ammonium hydrogen carbonate pH 8.0. Eluted fractions were vacuumed dried before analysis by 1-D electrophoresis.

EXAMPLE 11

Enzyme immunoassay for specific IgE and IgG_1 to recombinant proteins

Antigen-specific IgG1 and IgE were detected by ELISA as described previously (Shaw et al 1998a). The optimal concentration of soluble recombinant for coating the plates with Tco-Aspin having the amino acid sequence SEQ ID NO. 1 was determined to be 0.2 and 5 $\mu g\ ml^{-1}$ in PBS for IgE and IgG1 assays respectively. To get an indication of antibody levels in field sheep, serum samples were obtained from AgResearch's selectively bred Romney sheep lines selected for high (susceptible) or low (resistant) faecal nematode egg count. Samples were also taken at the same time from an unselected control flock. Results were expressed as mean absorbance in O.D. units. For statistical analysis antibody O.D values were $\log_e$ transformed to normalise the distributions.

Specific IgE immunoassay method:

Serum samples were pre-treated by precipitating 500 µl of serum with 500 µl of 76% saturated ammonium sulphate in distilled water. Samples were vortexed for 10 sec, repeated at 15 min,

and then after 30 min centrifuged in a microcentrifuge (10 min at 13,000 rpm). The supernatant was collected and diluted 1:1 in distilled water plus 0.1% Tween 20 in preparation for assay. Wells of microtiter plates (Costar EIA/RIA plate (#9017), Corning Incorporated, Corning, NY, USA) were coated for 2 hours at 37°C with optimally diluted recombinant Tco-Aspin (0.2µg ml^{-1}, 100 µl) in PBS pH 7.2. Following 3 washes with 0.05% (v/v) Tween 20 in distilled water (water-T20), plates were blocked for 5 min in 5% skim milk powder in PBS. Plates were washed 6 times in water-T20. Microtiter plates were then incubated with precipitated serum supernatant (100 µl per well in duplicate) overnight at room temperature. Following 6 washes in PBS-Tween 20, plates were incubated sequentially with mouse monoclonal antibody to ovine IgE (1/15 culture supernatant XB3/YD3, Shaw et al, 1998), goat anti-mouse gamma chain specific peroxidase conjugate (Sigma, St. Louis, Mo, USA). The enzyme substrate 3,3'5,5' tetramethylbenzidine was added and incubated for 30 min. The reaction was stopped 2N H_2SO_4. Plates were read using a 450 nm filter. A sheep was deemed Tco-Aspin IgE positive if its mean absorbance value was greater than 6 times the mean of assay blank wells plus 2 standard deviations.

EXAMPLE 12

Skin Testing

Skin testing was performed on AgResearch's 2002 born selectively bred Romney sheep lines. Monthly skin testing and serum sampling occurred from weaning until lambs were around 6 months of age (April 2003). Sheep were injected intradermally at either of two sites, the relatively wool-free area of the inside thighs, or occasionally the flank (after clipping of wool). Native or recombinant Tco-Aspin (0.1-10 µg) in PBS was injected using 26 gauge needles. Wheal and flare reactions were observed and measured approximately 30 min after injection. For the majority of the skin testing, 0.15-0.3 µg of Tco-Aspin was injected. The injection site located on the inside upper rear leg area has minimal wool coverage which aided injection and reaction evaluation. Using a tuberculin syringe with a 26 gauge x ½ inch needle, 100 µl of PBS containing Aspin was injected intra-dermally. Typically if injected correctly, a small distinctive bubble formed at the injection site. The time and sheep tag number was then recorded.

Injection of other animals then continued for around 20 minutes. Reading of reactions was carried in the order of injection. The sheep were located, caught, turned over and injection site identified. Reactions were scored on a scale of 0-4 (Table 1). A score of 0 or 1 is regarded as negative. A score of 2 or more is regarded as positive.

Table 1. Value and description of scoring used to assess skin testing reaction.

Score	Description
0	No reaction
1	Slight swelling only (no reddening)
2	Large swelling & no reddening
3	Swelling, and reddening
4	Large swelling, red and heat

The reaction was measured using a vernier caliper by reading the longest diameter of the reaction wheal and its transecting diameter, in millimeters. The area of skin test reaction was calculated by multiplying these 2 values (mm^2)

EXAMPLE 13

Pepsin inhibition assay

The inhibition of haemoglobin digestion activity of porcine pepsin (Kageyama 1998) was used to determine activity of native and recombinant Tco-Aspin. Pepstatin was used as a positive control.

Results and discussion

To identify allergenic proteins, TcL3-homogenate was separated electrophoretically, electroblotted and immunostained with affinity purified ovine IgE. Previous studies using 1-D SDS-Page gels had identified protein bands at approximate molecule weights of 12-14, 20-21, 27-31, 31-32 and 42 kDa as being associated with allergic immunity to *T. colubriformis* infections in field grazing sheep (Figure 1). Using 2-D electrophoresis technology, a protein spot corresponding to a strong IgE binding spot on Western blots with molecular weight 33,000 and pI 5.1 (Tco-Aspin) (Figure 2) was cut out from Coomassie blue stained 2-D gel (Figure 3) and analysed to determine amino acid sequence information. No positive match to known proteins was obtained using peptide mass fingerprinting with peptide masses obtained from the 33kDa/pI 5.1 spot. De novo peptide sequencing was possible from 2 tryptic peptides obtained from the isolated spot. These are; TC41A; 884.9 $[M+2H]^{2+}$ SASEQQE[L/I]TNYEK and TC41I; 737.46 $[M+3K]^{3+}$ GEAEQFL. A N- terminal sequence of LTVGTI was obtained by Edman degradation of the 33kDA/pI 5.1 spot following its transfer to a PVDF filter.

Preparation of recombinant Aspartyl protease inhibitor (Tco-Aspin)

Forward and reverse degenerate oligonucleotide primers were designed from the internal peptide sequences and used in PCR in combination with each other or with primers corresponding to the nematode splice leader sequence and polyT. A PCR product from *T. colubriformis* cDNA was obtained using the primers TC41Arev and SL1. This product was TA cloned (InVitroGen) into the plasmid pCR2.1 and the insert was sequenced. Analysis of the sequence revealed that this cDNA was from the same gene as a partial cDNA clone previously sequenced from a *T. colubriformis* cDNA library (unpublished). The overlapping, combined cDNAs encode a protein (Tco-Aspin) with strong homology to proteins of the aspartyl protease inhibitor family that contains the peptides identified by sequencing of the 33kDa/pI 5.1 spot (see below). The primers TC41.sig.5.nde and TC41.not.3 were synthesized based on DNA sequences at the amino- and carboxyl terminus of the Tco-Aspin coding region. These primers were used in a PCR with a *T. colubriformis* cDNA template to obtain a complete copy of the coding DNA for Tco-Aspin. The resultant PCR product was sequenced and cloned into the expression vector AY2-4 containing the arabinose promoter, a polyHis domain, and a monoclonal epitope tag. The plasmid was transfected into *E. coli* bacteria and successful

transfectants were identified by ampicillin resistance. The recombinant bacteria were grown in culture and induced for expression of Tco-Aspin with arabinose. Recombinant Tco-Aspin was found in both the soluble protein fraction and in the insoluble, inclusion body fraction following induction. The soluble Tco-Aspin was directly purified by immobilised metal affinity chromatography (IMAC). The insoluble fraction was solubilised in a chaotropic agent and then purified by IMAC.

Aspartyl protease inhibitor (Tco-Aspin)

This cDNA sequence of 684 base pairs (SEQ ID NO. 2) codes for a protein of 228 amino acids (SEQ ID NO. 1) with a predicted molecular mass of 25,414 Da and a calculated pI of 5.31. The N-terminus amino acid sequence contains a putative signal peptide (SignalP V1.1; http://www.cbs.dtu.dk/services/SignalP/index.html) with a suggested cleavage site between residues 15 and 16 (alanine-alanine). N-terminal sequencing showed that the mature protein's N-terminus is LTVGTI suggesting that further processing removes the amino acids APRQKR. The putative mature protein of 215 amino acids, without the signal sequence and amino acids APRQKR, would have a predicted molecular mass of 23056.77 Da and pI of 4.93. The sequence does not contain predicted N-glycosylation sites.

Computer searches with the predicted amino acid sequence revealed significant homologies with aspartyl protease inhibitors of *Ostertagia ostertagi* (CAD10783): (% identity/% similarity) (86%/90%), *Parelaphostrongylus tenuis* (AAG50205): (71%/83%), *Caenorhabditis elegans* (AAC46663): (50%/68%), *Acanthocheilonema viteae* (S23229): (40%/59%), *Dirofilaria immitis* (AAA70419): (42%/57%), *Onchocerca volvulus* (AAA29419): (42%/60%). A Clustal sequence alignment of Tco-Aspin with other members of the putative aspartyl protease inhibitor family is shown in Figure 4. These proteins share more distant homology to a known aspartyl protease inhibitor from the intestinal nematode *Ascaris suum* (Martzen et al, 1990). Structural features common to the nematode Aspins include the presence of a signal peptide sequence and the conservation of all four cysteine residues in the mature protein. The YVRDLT sequence motif, suggested as being of crucial functional importance in several filarial nematode inhibitors (Willenbucher J et al. 1993), is not well conserved in Tco-API-1 as this protein retains only a

shortened RDL motif at the equivalent position. Related inhibitors from *O. volvulus*, Ov33 (Tume et al. 1997) and *A. suum*, PI-3 (Martzen etal, 1990, Kageyama 1998) inhibit the in vitro activity of aspartyl proteases such as pepsin and cathepsin E. Using porcine pepsin, we were able to demonstrate a similar inhibitory activity with affinity purified native Tco-Aspin.

Rabbit antiserum to recombinant Tco-Aspin was applied to 1-D blots of TcL3 and Tc-adult homogenate (Figure 5). This identified strong staining bands in TcL3 homogenate at 31 and 22 kDa. Considerably weaker bands of the same molecular weights were seen on the adult homogenate. When rabbit antiserum produced to Tco-Aspin was applied to a 2-D blot of TcL3 homogenate, a series of closely placed spots of 32.2-34.5 kDa and pI 4.9-5.7 (Figure 6) were identified. These correspond in localisation to the original spot submitted for amino acid sequencing. A spot at 58.8 kDa, pI 5.6 is thought to be non-specific binding. An additional spot at 22.9 kDa, pI 5.7 was stained with rabbit anti Tco-Aspin antiserum and considered to be a breakdown product of Tco-Aspin.

Affinity purified native Tco-Aspin isolated from crude TcL3-homogenate and run on 1-D SDS-page gels (Figure 7) ran as 2 bands of molecular weight 31.3 and 21.8 kDa.

The 22 kDa antigen that was identified by probing of a 2-D blot of crude TcL3-homogenate, and that was affinity purified from the same material with rabbit anti-Tco-Aspin antibody, is probably a truncated product of Tco-Aspin. Attempts to identify this spot (see Figures 3 & 4: square) by amino acid sequencing techniques failed to produce a result due to insufficient amount of protein in gels. An abundant neighbouring protein positioned below the 22 kDa Tco-Aspin truncated product on 2-D gels was submitted for amino acid sequencing. Following sequencing of the *T. colubriformis* DNA coding for this protein, it was shown as having homology to a *Caenorhabditis elegans* hypothetical protein Y5F2A.1. No significant IgE binding could be demonstrated to a recombinant version of this protein. Approximately 40 µg of affinity purified native Tco-Aspin, was concentrated and loaded onto to a 1-D SDS-page gel. The gel was transferred to PVDF and stained to identify affinity purified products (Figure 8). Along with the 31 and 22 kDa protein bands, a third lower molecular weight band was also identified. Each of these three bands were excised and submitted for N-terminal sequencing. Edman sequencing

revealed that each had the same N-terminal amino acid sequence of LTVGTI. This shows that these proteins are truncated products of Tco-Aspin, likely generated by proteolytic cleavage at the C-terminal end and that the N-terminal end of Tco-Aspin is not degraded. Thus we conclude that the 20-21 kDa band identified by screening of sheep developing immunity to nematode infections (Figure 1) is a truncated product of Tco-Aspin.

In an effort to prepare a recombinant version of the 20-21 kDa truncated Tco-Aspin (Tco-Aspin*) an expression vector was generated expressing a Tco-Aspin peptide lacking the sixty-five amino acids C-terminal to the double lysines at position 164-165 of the protein sequence. The molecular weight of this peptide is proportional to that of native Tco-Aspin truncated product (20-21 kDa).

In developing an ELISA for Tco-Aspin, maximal reactivity with purified IgE from immune sheep was achieved with either soluble Tco-Aspin, or, after reduction, with both soluble and insoluble forms. Optimal protein concentration for coating microtitre plates was found to be 0.2 μg ml^{-1} for specific IgE assays and 2.5 μg ml^{-1} for specific IgG_1 assays using soluble Tco-Aspin. *Ostertagia circumcincta* (Oc-Aspin) (SEQ ID NO. 5), *Haemonchus contortus* (Hc-Aspin) (SEQ ID NO. 7) and truncated Tco-Aspin* (SEQ ID NO. 3) recombinant Aspins were also produced. Optimal protein concentration for specific IgE assays was found to be 0.25 μg ml^{-1} for Oc-Aspin and Tco-Aspin* and 5 μg ml^{-1} for Hc-Aspin.

Immunoreactivity of selection line sheep toward recombinant allergen

Specific IgE response to Tco-Aspin in nematode-infected sheep

IgE and IgG1 responses to recombinant Tco-Aspin were measured in lambs from lines selectively bred for resistance (R) or susceptibility (S) to nematode parasite infection at AgResearch's Wallaceville Animal Research Centre (Morris et al., 2000). An unselected control (C) line was also maintained. Lambs from all three lines grazed together on worm-infected pasture. Table 2 shows that the R-line lambs had significantly higher levels of anti-Tco-Aspin IgE than did S- or C-line lambs in serum samples taken in January and March when the lambs were approximately four and six months of age. In contrast, anti-Tco-Aspin IgG1 levels were

significantly different only between the R and S lines of sheep. These results show that specific IgE reactivity in lambs to the nematode allergen Tco-Aspin as early as 4 months of age can be used to identify animals that are better able to develop effective nematode worm immunity. 49% of R-line sheep were Tco-Aspin IgE positive in January and this increased to 58% by March. By contrast, 3% and 5% of S-line lambs, and 18% and 28% of the control line, respectively, were positive in January and March. Very similar results were obtained for R-line samples when they were assayed on native Tco-Aspin (data not shown).

Table 2

Mean specific antibody results in January or March (Optical Density values, back-transformed) for Wallaceville Selection Line male lambs born in 2001.

Sheep Line	*Tco*-API-1 IgE Jan	*Tco*-API-1 IgE Mar	*Tco*-API-1 IgG_1 Jan	*Tco*-API-1 IgG_1 Mar	Number of sheep
Susceptible	0.001^a	0.049^a	0.559^b	0.300^a	37
Controls	0.022^a	0.047^a	0.641^{NS}	0.471^{NS}	49
Resistant	0.200	0.390	0.688	0.529	51

Test for Line difference from Resistant-Line sheep, where ^{a} $P<0.001$; ^{b} $P<0.05$; ^{NS} Not significant.

Consistent with the selection-line differences in Table 2, a strong negative genetic correlation (Table 3) was found between log transformed Tco-Aspin (IgE) and log transformed faecal egg count (FEC). For example, the genetic correlation of $\log_e$Tco-Aspin January (IgE+1) with $\log_e$(FECJanuary+100) was 0.44 (s.e. 0.12; $P < 0.001$), and with $\log_e$(FECMarch+100) was 0.51 (s.e. 0.12; $P < 0.001$). (The genetic correlation indicates the degree to which genes controlling the expression of one performance measure in an animal are also associated with the expression of another performance measure.) No significant genetic correlations were found between specific IgE response to Aspin and dag scores, and (not shown) no significant genetic correlations were found between anti-Tco-Aspin IgG1 levels and FEC at four or six months of age, or between anti-Tco-Aspin IgG1 levels and dag scores.

Table 3. Genetic correlations between measured antibody traits, skin-test traits and $\log_e$ Faecal Egg Count (FEC), breech soiling score (dag score) or live-weight gain (weaning to January, or January to April).

	Genetic correlation with					
	$\log_e(FEC_{Jan}+100)$	$\log_e(FEC_{Mar}+100)$	Dag score 1 (Jan)	Dag score 2 (Mar)	Gain (Wng to Jan)	Gain(Jan to Apr)
$\log_e$(*Tco*-API-1 IgE $_{Jan+1}$)	-0.44 s.e. 0.12[a]	-0.51 s.e. 0.12[a]	0.18 s.e. 0.16[NS]	0.02 s.e. 0.16[NS]	-0.25 s.e. 0.17[NS]	-0.15 s.e. 0.18[NS]
$\log_e$(*Tco*-API-1 IgE $_{Mar+1}$)	-0.37 s.e. 0.09[a]	-0.44 s.e. 0.09[a]	0.11 s.e. 0.12[NS]	-0.04 s.e. 0.12[NS]	-0.13 s.e. 0.13[NS]	0.02 s.e. 0.14[NS]
Skin test score (April)	-0.04 s.e. 0.14[NS]	-0.16 s.e. 0.15[NS]				
Negative/Positive score (April)	-0.08 s.e. 0.16[NS]	-0.12 s.e. 0.17[NS]				
$\log_e$(Area $_{April+1000}$)	-0.00 s.e. 0.15[NS]	-0.15 s.e. 0.15[NS]				

[a] $P<0.001$

[NS]- not significant

It was then hypothesised that Tco-Aspin and perhaps aspartyl protease inhibitors from other gastro-intestinal nematodes, might have efficacy in an intradermal skin test aimed at rapidly identifying sheep disposed to develop immune resistance to nematodes under field conditions. Table 4 shows that, from an evaluation of the skin test with 218 selection-line animals, those in the R, C and S lines responded significantly differently to the skin test, whether measuring allergic area, using a 0-4 score, or defining a negative/positive criterion for allergy. These three measures were also significantly negatively associated with FEC measured in six-month animals ($P < 0.063$ for area, and $P < 0.005$, at least for test score (0-4) and negative/positive test outcome). Table 4 also shows that the R-line animals were not significantly different in response from the resilient-line lambs (the latter being bred for their ability to continue growing without the need for anthelmintic treatment when under serious nematode challenge).

Table 4. Summary of least squares analyses on skin-test traits in ewe lambs, using area measurement, test score, and negative/positive response.

Trait name	No	Sig. effects	Line	Back-transformed LS mean area	Transformed LS mean area ± s.e.	Log FEC2 cov. [a]
Log_e (Area+1000)	67	Line, P < 0.0004	81 (Resistant)	249	7.130 s.e. 0.013	
	58		2 (Control)	181 **	7.074 s.e. 0.014	
	41		82 (Susceptible)	167 ***	7.062 s.e. 0.016	
	52		85 (Resilient)	245 n.s.	7.127 s.e. 0.014	
	218					P<0.063
					LS mean	
Test score (0 to 4)	67	Line, P < 0.0003	81 (Resistant)		1.866 s.e. 0.124	
(Not transformed)	58		2 (Control)	**	1.328 s.e. 0.133	
	41		82 (Susceptible)	***	1.122 s.e. 0.158	
	52		85 (Resilient)	n.s.	1.807 s.e. 0.140	
	218					P<0.004
					LS mean	
Negative/Positive	67	Line, P < 0.0005	81 (Resistant)		0.642 s.e. 0.059	
(Neg test=scores 0, 1;	58		2 (Control)	**	0.362 s.e. 0.064	
Pos test=scores 2 - 4)	41		82 (Susceptible)	***	0.317 s.e. 0.076	
	52		85 (Resilient)	n.s.	0.596 s.e. 0.067	
	218					P<0.005

[a] Correlation (covariance) with log (FEC2) in Lines 2, 81 and 82 only;

** $P < 0.01$; *** $P < 0.001$.

These results extend previous work in which we reported a negative genetic correlation between FEC and IgE responses of R- and S-line lambs exposed to nematode challenge while grazing (Shaw et al.,1999). However, unlike the previous work where specific IgE to a crude mixture of TcL3 ES antigens showed a positive genetic correlation to the level of breech soiling ("dag score") in March, there was no such correlation between Tco-Aspin specific IgE and dag score in the present study. This suggests that selection of sheep for resistance to parasite infection, based on increased specific IgE levels to Tco-Aspin, should not lead simultaneously to increased breech soiling. The formation of dags in the breech area is brought about by diarrhoea thought to be associated with inflammatory responses to nematode infections and is particularly evident on some resistant-line sheep (Bisset et al., 2001). We have thus confirmed that it is possible to establish more practical tests to measure IgE levels specific for defined allergens, apart from ELISA, and these may be used effectively as part of a selective breeding program.

Animal resources used in the studies for Tables 2- 4:

Table 2: (Least squares analysis)_137 born-2001 Resistant (R), Susceptible (S) & Control (C)-line ram lambs.

Table 3: (Genetic correlations from ASREML analyses)

Rows 1 & 2: ELISA results for b'89-91 (n=709) Progeny-Test lambs, plus b'2001 (n=592) lambs, with all FEC and Dag-score data at Wallaceville from 1979-2001. (N.B. no ASPinIgE(Jan) for b'89 Prog Test lambs; only March)

Rows 3-5: Skin test results for b'2002 lambs (223 Ewe lambs from all lines of ewe lambs at Wallaceville (Lines 2, 22, 42, 81, 82 & 85), plus 42 R-line ram lambs, with all FEC data from 1979-2002).

Table 4: (Least squares analysis) 218 ewe lambs from R, S, C and Resilience lines.

Comparison of Tco-Aspin Skin Test with specific IgE ELISA

Score correlation with ELISA: The association between skin test score, negative (score 0-1) or positive (score 2-4) and ELISA negative or positive (sample rated IgE positive if its mean absorbance value was greater than the mean of assay blank wells plus 2 standard deviations) was determined using Chi square test.

Table 5a. Ram lambs significance of association between score Neg/Pos and ELISA at different cut-offs (1 to 10 times the mean of the assay blank wells plus 2 standard deviations (Bkgd))

Bkgd x	16/12/02	21/01/03	25/02/03	18/03/03	17/04/03
1	0.153	1.000	1.000	1.000	1.000
2	0.008 [a]	0.007 [a]	0.003 [a]	0.113	1.000
3	0.042 [a]	0.000 [a]	0.002 [a]	0.049 [a]	0.143
4	0.018 [a]	0.001 [a]	0.006 [a]	0.022 [a]	0.017 [a]
5	0.328	0.000 [a]	0.002 [a]	0.004 [a]	0.004 [a]
6	0.328	0.000 [a]	0.000 [a]	0.029 [a]	0.040 [a]
7	0.172	0.000 [a]	0.000 [a]	0.036 [a]	0.040 [a]
8	0.172	0.001 [a]	0.000 [a]	0.007 [a]	0.013 [a]
9	0.172	0.000 [a]	0.000 [a]	0.003 [a]	0.008 [a]
10	0.060	0.003 [a]	0.000 [a]	0.003 [a]	0.002 [a]

[a] $P<0.05$

Table 5b. Ewe lambs significance of association between score Neg/Pos and ELISA at different cut-offs (1 to10 times the mean of assay blank wells plus 2 standard deviations (Bkgd))

Bkgd x	18/12/02	22/01/03	20/02/03	14/03/03	2/04/03
1	0.321	0.210	1.000	0.205	1.000
2	0.001 [a]	0.008 [a]	0.000 [a]	0.084	0.015 [a]
3	0.068	0.000 [a]	0.000 [a]	0.012 [a]	0.050
4	0.075	0.005 [a]	0.000 [a]	0.005 [a]	0.001 [a]
5	0.985	0.001 [a]	0.000 [a]	0.002 [a]	0.001 [a]
6	0.624	0.000 [a]	0.000 [a]	0.007 [a]	0.003 [a]
7	0.624	0.000 [a]	0.000 [a]	0.007 [a]	0.004 [a]
8	0.159	0.000 [a]	0.000 [a]	0.004 [a]	0.001 [a]
9	0.159	0.001 [a]	0.002 [a]	0.001 [a]	0.003 [a]
10	0.159	0.002 [a]	0.007 [a]	0.001 [a]	0.002 [a]

[a] $P<0.05$

Except at weaning (December), these results suggest that an ELISA value greater than four times the mean background value for an ELISA plus two standard deviations would be significantly associated with a positive skin test reaction.

The chi-square for scores (negative versus positive) falling in the top vs. bottom half of the rank list for ASP IgE (March) was 61.5 (i.e. P<0.0001). Table 6 shows that log(ELISA IgE) and log(Skin test) are closely correlated (P<0.0001) in samples taken on ewe or lambs at about 6 months of age.

Area correlation with ELISA: Prior to analysis, $\log_e$(Area measurement+100) and $\log_e$(corrected ELISA value + 1) were calculated. The skin test measurement was analyzed using least squares, with sex as a fixed effect and $\log_e$(ELISA IgE) for the relevant date as a covariate.

Table 6. Test of the association (significance test) between log(ELISA IgE) and log(Skin test area in mm^2 + 100)

Date	Sex	N Records	R^2 accounted for by model	Significance test*	Regression of log(area+100) on log (ELISA IgE)
2/4/03, 18/3/03	Ewe + Ram	109	0.17	P < 0.0001	0.79 ± - 0.17
17/4/03	Ram	42	0.43	P < 0.0001	0.65 ± - 0.11

We conclude that ELISA IgE and Skin Reaction Area are significantly associated

The selection line differences in 2002 born lambs (Resistant vs Susceptible) for Aspin IgE, both January and March, were highly significant (P<0.0001). The selection line differences in Area were significant (P<0.001)

Heritability of Aspin specific IgE ELISA and skin test

Table 7

	Heritability
Aspin IgE (January)	0.1802 se 0.054 $P < 0.001$
Aspin IgE (March/April)	0.347 se 0.0662 $P < 0.0001$
Skin test score (April)	0.3857 se 0.1735 $P < 0.05$
Skin test Neg/Pos (April)	0.2808 se 0.1638 Not Significant
Skin test Area (April)	0.3421 se .01757 $P < 0.053$

These results show that both the ELISA and skin test are heritable traits

Comparison of different nematode Aspins

Coding sequence for Aspins from *O. circumcincta* (Teladorsagia circumcincta) and *H. contortus* was obtained and recombinant proteins expressed. The coding DNA was inserted into bacterial expression vectors and the corresponding recombinant proteins were produced and purified as described previously for Tco-Aspin. The amino acid sequences of the three Aspins are very similar. Homologies with Tco-Aspin for *Ostertagia circumcincta* are (% identity/% similarity) (87.1%/89.8%) and *Haemonchus contortus* (84.9%/87.6%)

Serum samples from Resistant line ram lambs born in 2001 were used to compare the reactivity of IgE to various native and recombinant Aspin preparations (Table 8).

Table 8. Specific IgE reactivity of 2001 born Resistant line ram lambs (N=48) in March 2001 to various Aspin preparations

	Rec. Tco Aspin 1	Rec. Tco-Aspin 2	Rec. Tco-Aspin*	Native Tco-Aspin	Rec. Oc-Aspin	Rec. Hc-Aspin
Number positive	29	29	22	28	42	13
% positive	60.4	60.4	45.8	58.3	87.5	27.1

Native and recombinant Tco-Aspin identified around 58-60% of the resistant line ram lamb as Aspin IgE positive. Of those serum samples positive to native Aspin, recombinant Tco-Aspin and Oc-Aspin identified 96.4% and Hc-Aspin 42.9%. Oc-Aspin identified a further 27.1% serum samples as Aspin IgE positive. The truncated form of Tco-Aspin (Tco-Aspin*) identified 45.8% of resistant line ram lambs, 71.4% of those animals identified by native Tco-Aspin. This comparison between Tco-Aspin & Tco-Aspin* suggests that most of the IgE epitopes are found on the truncated form of Tco-Aspin. We conclude that recombinant Tco-Aspin is as effective as native *T. colubriformis* Aspin in identifying Aspin IgE positive animals. Recombinant *O. circumcincta* may be of better use in that it identifies a greater number of sheep as Aspin positive animals. *H. contortus* Aspin is less useful in identifying Aspin positive animals

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope of the appended claims.

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